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Latent membrane protein 1 of Epstein–Barr virus regulates death-associated protein kinase 1 in lymphoblastoid cell line

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ABSTRACT

The Epstein–Barr virus (EBV) infects and transforms primary B cells into lymphoblastoid cell lines (LCLs). We observed death-associated protein kinase 1 (DAPK1) upregulation in B cells following EBV infection and high DAPK1 levels in LCLs. DAPK1 participates in several apoptosis-inducing pathways, yet DAPK1 expression increased during B cell transformation. Data from LMP1 overexpression in LCLs and HeLa cells and from knocked down LMP1 in LCLs suggest LMP1 regulation of DAPK1 expression. We observed NF-κB signaling in DAPK1 upregulation by LMP1 with CTAR deletion mutants failing to induce DAPK1 expression and with Bay11 blocking DAPK1 expression. DAPK1 is inactive in LCLs due to insufficient stimuli, and is not regulated by Ser308 phosphorylation. However, DAPK1 in LCLs is functional, as evidenced by its quick mediation of cell death following UV or H_2O_2 exposure, and increased survival among LCLs knocked down with DAPK. DAPK roles in EBV-infected B cells remain to be identified.

Introduction

The Epstein–Barr virus (EBV) is capable of establishing life-long infections and maintaining latency in over 95% of immuno-competent adults (Babcock et al., 1998; Henle et al., 1969). In vitro, EBV immortalizes B cells into lymphoblastoid cell lines (LCLs), causing them to harbor episomal genomes and persistent infections (Kieff, 1996). Viral proteins expressed in LCLs include latent membrane proteins (LMPs) 1, 2A, and 2B, and Epstein–Barr nuclear antigens (EBNAs) 1 through 6, each with a distinct function (Kieff, 1996). EBNA1 maintains the episomal genome(Kieff, 1996), and EBNA2 (involved in immortalization) acts as a Notch receptor activating RBP-Jκ repressed promoters (Grossman et al., 1994; Henkel et al., 1994). LMP1, a major transforming protein, mimics a constitutively activated CD40-TNF (tumor necrosis factor) receptor (Eliopoulos and Young, 2001; Uchida et al., 1999). LMP1 controls both cell proliferation and death by exploiting the features of TNF receptors, which are considered crucial to cell growth, differentiation, and apoptosis (Gaur and Aggarwal, 2003; Pimentel-Muinos and Seed, 1999; Soni et al., 2007).

In vivo, EBV maintains latency to avoid immune system detection. A sudden change in equilibrium between immune control and virus–cell interaction can lead to the insufficient killing or overproliferation of virus-infected cells, resulting in lymphoproliferative syndrome in immunocompromised patients, Burkitt's lymphoma, or Hodgkin's disease (Middeldorp et al., 2003). LMP1 promotes cell proliferation and inhibits apoptosis—both important for immortalization (Kaye et al., 1993; Li and Chang, 2003). However, LMP1 is also capable of upregulating Fas and sensitizing cells to become pro-apoptotic (Dirmeier et al., 2005). These contradictory functions maintain EBV-infected cell homeostasis.

Death-associated protein kinase 1 (DAPK1) is a Ca^{2+}/calmodulin-dependent Ser/Thr kinase requiring regulation due to its status as a pro-apoptotic protein (Cohen et al., 1997). DAPK1 action is necessary to induce cell death via such stimuli as death receptors, cytokines, extracellular matrix detachment, or hyperproliferation due to oncogene activation (Cohen et al., 1999; Inbal et al., 1997; Jang et al., 2002; Pelled et al., 2002; Raveh et al., 2000). Due to its autoinhibitory mechanism, DAPK1 activation requires the dephosphorylation of Ser308 and Ca^{2+}/calmodulin binding (Shohat et al., 2001). Extracellular signal-regulated kinase (ERK) and mitogen activated protein kinase (MAPK) signaling

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pathways are also involved in DAPK1 action regulation (Anjum et al., 2005; Chen et al., 2005). Furthermore, respective Tyr491/492 phosphorylation or dephosphorylation by Src or leukocyte common antigen-related tyrosine phosphatase (LAR) reciprocally modulates DAPK1 function (Wang et al., 2007).

In this study, we identified a cellular protein DAPK1 which is upregulated in EBV-infected B cells and LCLs. Since DAPK1 is pro-apoptotic, we further investigated its regulation in B cells. The tight regulation on DAPK1 activation ensures cell survival and may play a role in maintenance of EBV latency in B cells.

Results and discussion

To study EBV gene expression profile in LCLs, we purified primary $CD19^+$ B cells using magnetic bead separation from leukocyte concentrates and infected them with the B95-8 EBV virus. Total RNA was extracted from cells harvested 0, 7, 14, and 28 days post-infection and used for microarray analyses; DAPK1 was among the highly induced genes we observed. Real-time PCR and immunoblotting analysis were used to confirm microarray results. As shown in Fig. 1A, gradual increases in DAPK1 expression were detected on days 3, 7, 14, 21 and 28, and high DAPK1 levels were maintained in lymphoblastoid cell lines following normalization to DAPK1 levels in uninfected B cells (day 0) (Fig. 1B). Very little change in DAPK1 expression was noted in B cells that were either cultured for 2 days without infection or stimulated with PMA. Similar protein upregulation data were produced by immunoblotting. EBV infection was confirmed by EBNA1 expression (Fig. 1C).

As one EBV latent protein, LMP1 affects broad cellular gene expression, especially among cell growth genes (Dirmeier et al., 2005). Because hypermethylation exists in the DAPK1 promoter region in most B cell lines, we used epithelial cell lines to investigate links between LMP1 and DAPK1. We transfected LMP1 plasmid in HeLa cells and used a combination of RT-PCR and immunoblotting to analyze DAPK1 expression. As shown in Fig. 2A, DAPK1 was upregulated following LMP1 overexpression. LMP1 transient transfection in two LCL cell lines also resulted in DAPK1 upregulation (Fig. 2B). In contrast, reduced LMP1 expression by siRNA knockdown decreased DAPK1 expression (Fig. 2C). Since carboxy-terminal activating regions 1 and 2 (CTAR1 and CTAR2) are responsible for LMP1 signaling (Huen et al., 1995), we looked at the potential effects of CTAR-deleted LMP1 mutants on DAPK1 expression. DAPK1 expression was not increased after HeLa cells were transfected with various CTAR mutants, especially the CTAR2 deletion and double CTAR deletion mutants (Fig. 2D). This suggests that CTAR2 has greater importance in terms of LMP1 signaling in DAPK1 expression regulation.

We used several inhibitors to determine the downstream signaling pathway responsible for DAPK1 induction by LMP1. LCLs showed decreased DAPK1 levels following treatment with Bay11, which specifically inhibits the NF-κB pathway (Fig. 3A). The addition of 35 μM of Bay11 reduced DAPK1 levels in LMP1-expressing HeLa cells; higher concentrations of Bay11 significantly decreased DAPK1 expression (Fig. 3B). According to these findings, DAPK1 is positively regulated by the NF-κB pathway. To identify other signal transduction pathways for DAPK1 induction, we tested the MAPK inhibitors SP600125, SB203580, and PD98059. Even though these inhibitors respectively block JNK, p38, and ERK phosphorylation, in this experiment they failed to downregulate DAPK1 expression in LCLs (Fig. 3C). Combined with the CTAR deletion LMP1 mutant data, these results indicate that NF-κB is capable of mediating DAPK1 induction by LMP1 in both LCLs and HeLa cells, but MAPK signaling pathways are not.

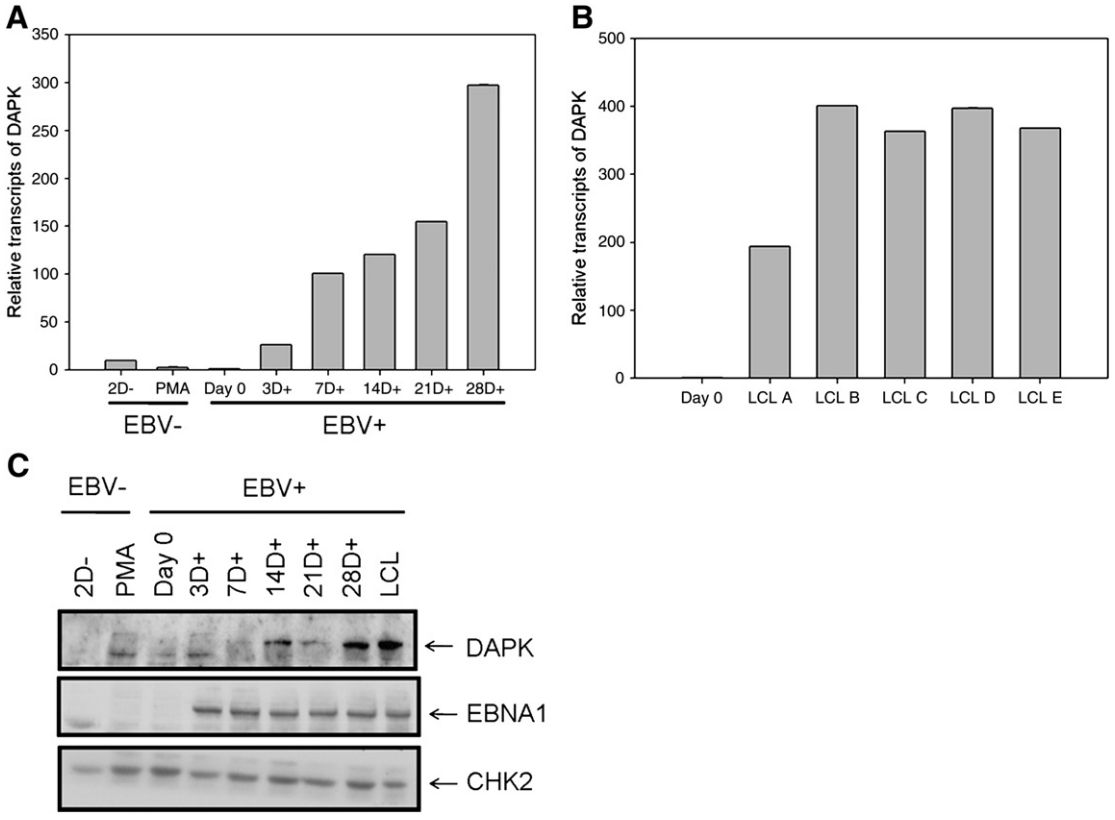

Fig. 1. Increased DAPK expression in EBV-infected B cells. (A) Purified B cells were infected with B95.8 strain EBV and harvested for total RNA extraction at different time points. DAPK expression was analyzed by real-time PCR. B cells cultured for 2 days without infection (2D−) and B cells treated with 25 ng/ml PMA (phorbol 12-myristate 13-acetate) for 2 days (PMA) are shown. Relative DAPK expression was normalized to day 0 B cells. Increased DAPK transcripts are noted in EBV-infected B cells. (B) DAPK transcript expression levels in five LCL lines. (C) Total cell lysates were prepared at different time points; total proteins (20 μg) were separated by SDS-PAGE and analyzed by immunoblotting with anti-DAPK and anti-EBNA1 antibodies (CHK2 as internal control).

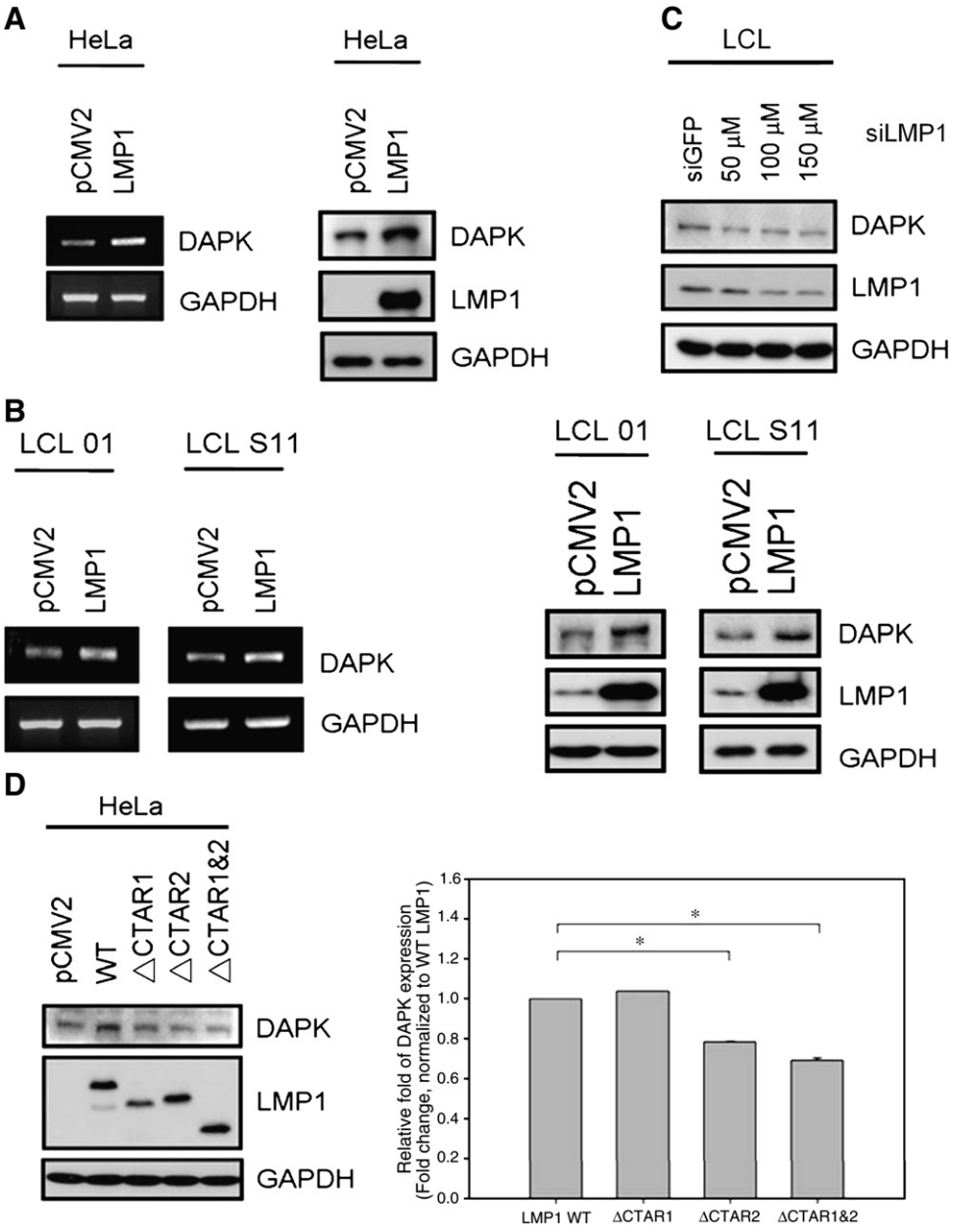

Fig. 2. EBV viral protein LMP1 mediates DAPK expression. (A) HeLa cells (5×10^5) were transfected with LMP1 expressing vector or pCMV2 for 18 h prior to total RNA extraction. RNA (2 μg) was used for RT-PCR analysis. Cell lysates were prepared 24 h post-transfection for immunoblotting. (B) LCL01 and LCLS11 cells (2×10^6 each) were electroporated with 8 μg of LMP1 expressing vector or pCMV2 control vector. Total RNA was extracted after 36 h; 2 μg were used for RT-PCR analysis. DAPK, LMP1 and GAPDH protein levels were determined by immunoblotting 48 h post-electroporation. (C) LCL cells (1×10^6) were electroporated with different concentrations of LMP1-siRNA or control GFP-siRNA. LMP1 and DAPK expression was determined by immunoblotting 48 h post-electroporation. (D) HeLa cells (4×10^5) were transfected with 4 μg of pCMV2, wild type LMP1 (LMP1 WT), or different CTAR-deleted mutants. Total proteins were collected at 24 h for DAPK detection by immunoblotting. DAPK and LMP1 intensities were estimated using Quantity One software (Bio-Rad Laboratories, Hercules, CA) and calculated as relative folds of expression (see histogram). Data are presented as mean ± SD (*$p<0.05$, two-tail Student's *t*-test).

Based on the association between DAPK1 expression and cell death, we speculated that DAPK1 is inactive in LCLs. To test this idea we performed in vitro kinase assays to evaluate DAPK1 activity. As shown in Fig. 4A, Flag-DAPK1 overexpression and immunoprecipitation in HEK293T cells resulted in the phosphorylation of myosin light chains; no kinase activity was detected in endogenous DAPK1 immunoprecipitated with anti-DAPK1 antibodies in LCLs. Ser308 (S308) phosphorylation has been shown to inhibit DAPK1 catalytic activity (Shohat et al., 2001); however, in this study the S308 phosphorylation of DAPK1 in LCLs was barely detectable (Fig. 4B). This suggests that S308 phosphorylation does not account for the lack of DAPK1 activity in LCLs, therefore other regulatory mechanisms are likely involved.

Since DAPK1 is inactive, we tried to determine if it loses its biological function in LCLs. We used shRNA to knock down DAPK1 expression in LCLs, followed by PI (propidium iodide) staining and cell proliferation WST1 assays for evaluating cell viability in response to death signals following treatment with ultraviolet (UV) or H_2O_2 (Fig. 4C). Post-UV or H_2O_2 treatment, we observed lower cell death rates (Supplemental Data) and higher survival rates (Fig. 4D and E) in shDAPK1 clones (low DAPK1 expression). According to a previous report, H_2O_2-activated DAPK1 phosphorylates protein kinase D (PKD) at Ser910 (S910) and regulates JNK signals leading to cell death (Eisenberg-Lerner and Kimchi, 2007). We tried to determine if PKD is phosphorylated by DAPK1 under oxidative stress. As shown in Fig. 4F, p-PKD expression was detected by immunoblotting, indicating upstream DAPK enzymatic activity. According to these results, DAPK1 is functional in LCLs, since it plays a role in cell death associated with oxidation or irradiation stress.

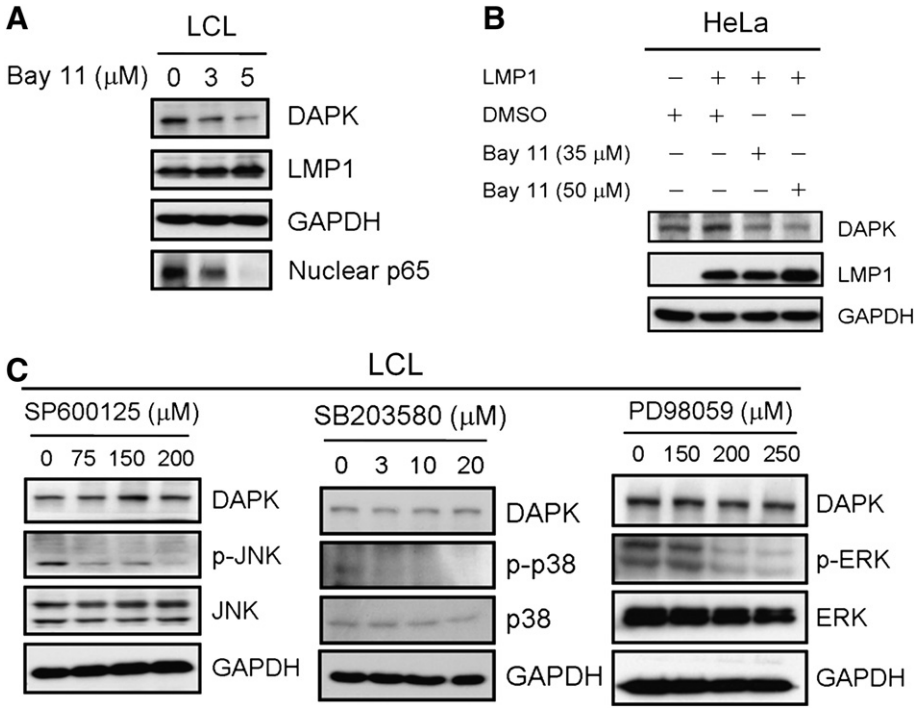

Fig. 3. DAPK expression in LCL is mediated by NF-κB signaling. (A) LCL cells (2×10^6) were treated with NF-κB inhibitor Bay11-7082 (0, 3 or 5 μM) for 24 h. Total proteins (20 μg) were separated by SDS-PAGE and immunoblotted with anti-DAPK, anti-LMP1, or anti-GAPDH antibodies. Nuclear protein (40 μg) was immunoblotted with anti-NF-κB p65 antibodies. (B) HeLa cells (4×10^5) were transfected with pCMV2 or LMP1 vectors and incubated at 37 °C for 24 h followed by treatment with 35 μM or 50 μM of Bay11-7082 or DMSO as control and cultured for 18 h. Total proteins were collected for immunoblotting. (C) LCL cells (2×10^6) were treated with different quantities of SP600125 (JUN inhibitor), SB203580 (p38 inhibitor), or PD98059 (ERK inhibitor) for 24 h. DAPK levels were compared by immunoblotting. Phosphorylated forms of MAPK (p-JNK, p-p38 and p-ERK) were used to demonstrate the inhibitory effects of MAPK inhibitors; JNK, p38, and ERK served as loading controls.

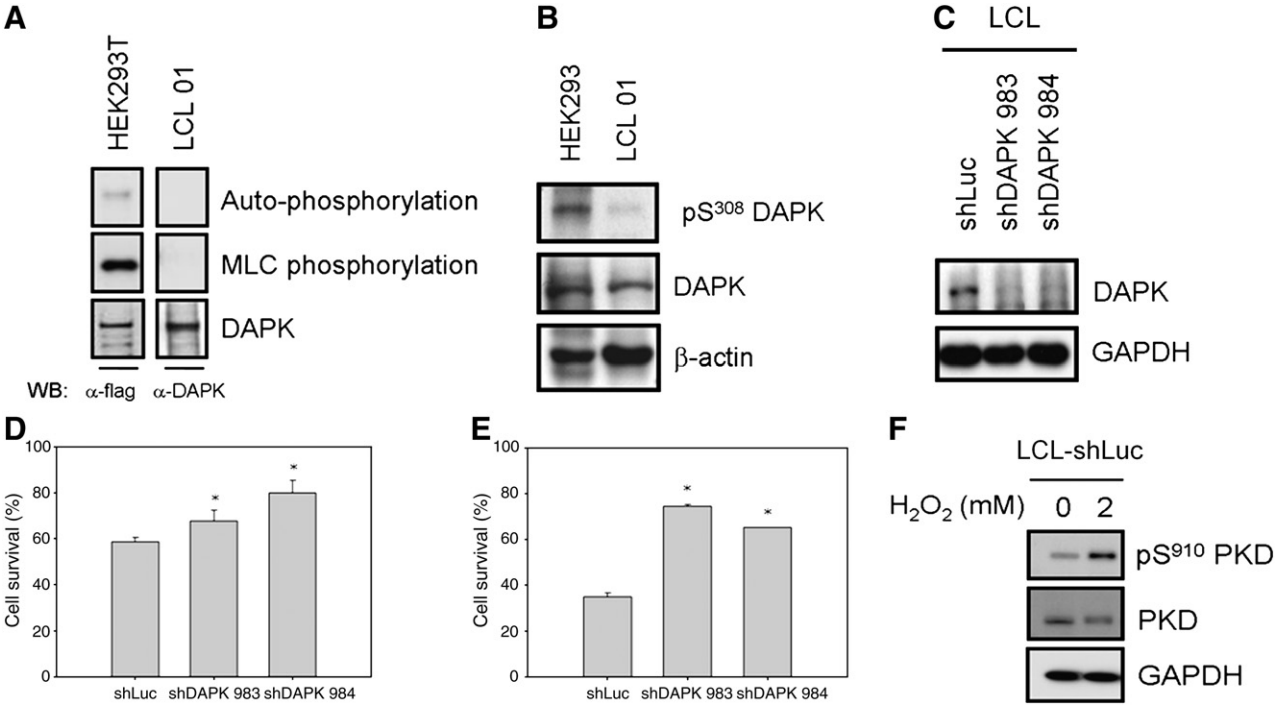

Fig. 4. DAPK in LCLs is inactive until subjected to stress. (A) Endogenous DAPK activity was determined by in vitro kinase assay. DAPK proteins were precipitated from LCLs and DAPK-expressing HEK293T cell lysates, and incubated with MLC and γP^{32}-ATP at room temperature for 15 min. Autoradiography was performed to check kinase activity. DAPK protein levels are also shown. (B) Total proteins from HEK293 and LCL (20 μg each) were blotted with anti-DAPK and anti-DAPK pS308 antibodies. (C) DAPK expression was knocked down in shDAPK983 and shDAPK984. shLuc cells served as a negative control. 2×10^6 cells were stimulated in the presence or absence of UVC exposure (254 nm) (D) or treated with 1 mM H_2O_2 (E). UV-exposed cells and H_2O_2-treated cells were collected at 18 h and 24 h, respectively. Cell survival was determined by WST-1 proliferation assay. Survival percentage was calculated as O.D. 450 absorbance in each batch of treated cells divided by absorbance in untreated cells. Results are expressed as mean ± SD (*$p<0.05$, two-tail Student's *t*-test). (F) Endogenous DAPK gains enzymatic activity to phosphorylate downstream PKD substrate in H_2O_2-treated LCL cells. LCL-shLuc cells (2×10^6) were left untreated or treated with 2 mM H_2O_2 for 24 h. Total proteins (20 μg) were immunoblotted with anti-PKD and anti-PKD pS910 antibodies.

We have tried HeLa cell transfection with different viral protein vectors, and found that only LMP1 mediates DAPK1 upregulation (data not shown). LMP1 is the primary viral protein responsible for B cell transformation and proliferation via NF-κB signaling (Li and Chang, 2003; Soni et al., 2007). In addition to stimulating cell growth, LMP1 also upregulates anti-apoptotic molecules such as Bcl-2, A-20, and Mcl-1 (Fries et al., 1996; Henderson et al., 1991; Laherty et al., 1992; Wang et al., 1996). However, note that LMP1 is still capable of (a) upregulating Fas expression in LCLs recognized and killed by FasL expressing cytotoxic T cells and (b) promoting apoptosis in some cells (Falk et al., 1992; Larochelle et al., 1998; Le Clorennec et al., 2006). According to a previous report, LMP1 overexpression in LCLs induces apoptosis via Fas and caspase 8 (Le Clorennec et al., 2008). The same researchers suggest that LMP1-dependent NF-κB signaling (which mediates Fas overexpression) may counteract the anti-apoptotic effects of NF-κB. Evidence has also been gathered indicating that NF-κB is a mediator of LMP1-induced cell death (Nitta et al., 2003). It may be that NF-κB signaling in LCLs results in apoptosis triggered by pro-apoptotic stimuli beyond the control of anti-apoptotic factors.

There appears to be a correlation between LMP1 effects and expression level. As demonstrated in epithelial cells (Lu et al., 1996) and B cell lines (Floettmann et al., 1996), increased LMP1 levels tend to result in cell growth arrest or apoptosis. LMP1 function (which promotes cell proliferation at normal levels and induces cell death at high levels) is similar to that of c-myc. p53 induction is associated with LMP1 and c-myc overexpression (Le Clorennec et al., 2006). The contradictory functions of the same protein may act as a switch for self-protection in the form of preventing the excessive growth of EBV-infected cells. LMP1 levels are increased by the JNK pathway but decreased by NF-κB activation (Goormachtigh et al., 2006). It has been reported that EBV not only immortalizes B cells but also sensitizes infected B cells for elimination by cytotoxic T cells (Le Clorennec et al., 2008). Despite the importance of Fas upregulation by LMP1 via NF-κB, we believe DAPK1 may be another cellular protein target induced by similar pathways, one playing a role similar to that of Fas. The balance between cell proliferation and the expression of pro-apoptotic proteins dependent on LMP1 and NF-κB levels may be the basis for EBV-immortalized B cell confinement by the immune system.

In most tumors, DAPK1 expression is silenced due to promoter hypermethylation, yet still expressed in human renal cell carcinomas (RCC) and acute myelogenous leukemia (Guzman et al., 2001). Results from in vitro kinase assays indicate that RCC cell lines overexpress DAPK1 without detectable activity (Wethkamp et al., 2006). We found that LMP1 activation is capable of inducing the expression of the pro-apoptotic DAPK1 protein. This protein quickly responds to oxidative stress or UV stimulation by promoting programmed cell death. Identifying DAPK1 expression in LCLs is a possible mechanism for the homeostatic control of in vivo latency.

Conclusions

In summary, our results indicate that DAPK1 is gradually upregulated in EBV-infected primary B cells and maintains high expression levels in LCLs. We also found that LMP1 is involved in DAPK1 induction, and that NF-κB signaling participates in this upregulation. In addition, we determined that DAPK1 remains inactive under normal conditions, and is activated by external stimuli such as UV or oxidative stress.

Materials and methods

Cell culture and EBV infection

EBV B95.8 strain was prepared prior to B cell infection. 40 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (TPA)(Sigma-Aldrich, St. Louis, MO) and 3 mM sodium butyrate (Sigma-Aldrich) were added to B95.8 cell culture medium and incubated at 37 °C for 72 h. Viral particles in supernatant were collected by centrifugation and concentration. After 0.45 μm filtration (Millipore, Billerica, MA), viruses were aliquoted and stored at −80 °C. Total B cells were purified by Ficoll-Paque (GE Life Sciences, Uppsala, Sweden). Human peripheral blood mononuclear cells (PBMCs) were separated with EasySep human CD19 selection cocktail (StemCell Technologies, Vancouver, Canada) following the manufacturer's instructions. Purified B cells (2×10^6/well) were infected with B95.8 strain EBV in a 12-well plate. Infected B cells were maintained for at least 28 days to produce lymphoblastoid cell lines (LCLs). One LCL 01 and one LCL S11 strain were developed from different blood donors. Infected B cells and LCLs were cultured in RPMI medium (Gibco, Carlsbad, CA) containing 10% FBS (Gibco) and 1% Pen-Strep antibiotic solution (Biological Industries, Kibbutz Beit Haemek, Israel). HeLa, HEK293T, and HEK293 cells were grown in DMEM medium (Gibco) with 10% FBS (Gibco). NF-κB inhibitor Bay11-7082, JNK inhibitor SP600125, p-38 inhibitor SB203580, and ERK inhibitor PD98059 were purchased from Merck (Darmstadt, Germany).

Plasmids and gene transfection

The LMP1 EBV viral protein expression vector and pCMV2 control vector (both provided by Dr. Y.-S. Chang, Chang Gung University, Taoyuan, Taiwan) were overexpressed in LCLs and HeLa cells by electroporation and transfection, respectively. Plasmids (4 μg) were transfected into adherent HeLa cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. For LCLs, 2×10^6 cells were electroporated with 8 μg of plasmids using a MicroPorator (NanoEnTek, Seoul, South Korea) (1375 V, 30 ms, 1 pulse). Plasmids with LMP1 CTAR-deleted mutants were provided by Dr. C.-J. Chen (National Yang-Ming University, Taipei).

RT-PCR and real-time PCR

Total cellular RNA from LCL and HeLa cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from 2 μg of total RNA by reverse transcription with a RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada) according to the manufacturer's instructions. To detect DAPK expression, PCR was performed with RT products, primers, dNTP, and Taq polymerase as follows: 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min (30 cycles total). Primers for DAPK amplicons (496 bp) were 5'-TGCGGCCAA CAACGGAATC-3' (forward) and 5'-GGGTCGGGGCCA CAAACAC-3' (reverse). GAPDH amplicons (598 bp) were amplified using the primers 5'-GGGTGTC GCTGTTGAA-3' (forward) and 5'-GCTGAGTACGTCGTGG-3' (reverse). To analyze DAPK transcripts in EBV-infected B cells with real-time PCR, RT products were mixed with Human Universal Probe Library Taqman probe No. 45 (Roche, Basel, Switzerland), forward primer 5'-TGCTGAAAGAGATTAGGAACAGG-3', reverse primer 5'-CGAAGTAC CTTCATGTCCTTTGA-3', and FastStart Taqman Probe Master mix (Roche). Reactions were amplified with ABI PRISM 7900 as follows: 1 cycle of 50 °C for 2 min and 60 °C for 1 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Immunoblotting

Total cellular proteins were prepared directly from cell lysates. For nuclear protein extraction, cells were mixed with buffer I (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$), held on ice for 15 min, mixed with buffer II (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 2.5% NP-40), and held on ice for 5 min. After centrifugation, nuclear proteins from cell pellets were resuspended in buffer III (20 mM HEPES, 0.45 M NaCl, 1.0 mM EDTA), sonicated, and collected by additional centrifugation. 20 μg total proteins or 40 μg purified nuclear proteins were separated by SDS-PAGE, transferred to an Immobilon P membrane (Millipore,

Billerica, MA), and blotted with specific antibodies. Relative protein amounts were visualized on X-ray film in the presence of Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA). The following antibodies were used in this study: anti-DAPK and anti-DAPK pS308 (purchased from Sigma-Aldrich); anti-EBNA1 and CHK2 (provided by Dr. C.-H. Tsai, National Taiwan University); anti-LMP1 (provided by Dr. C.-J. Chen, National Yang-Ming University); and anti-GAPDH (purchased from Novus Biologicals, Littleton, CO). The following antibodies were used for signaling study: JNK, ERK1/2, and p-ERK1/2 (purchased from Millipore, Billerica, MA); and p-JNK, p38, and p-p38 (purchased from Santa Cruz Biotechnology, Santa Cruz, CA). Anti-PKD (Cell Signaling Technology, Danvers, MA) and anti-PKD pS910 antibodies (Abcam, Cambridge, MA) were used to analyze DAPK downstream substrate phosphorylation.

Small interfering RNA (siRNA)

Oligonucleotides corresponding to the B95.8 stain of EBV LMP1-specific siRNA duplexes were synthesized as sense 5'-GGAAUUUGCACG-GACAGGCUU-3' and antisense 5'-GCCUGUCCGUGCAAAUUCCUU-3' (2). Control siRNA was synthesized to target GFP expression. Duplex sequences were sense 5'-GCACGACUUCUUCAAGUCCUU-3' and antisense 5'-GGACUUGAAGAAGUCGUG CUU-3'. LCL cells (1×10^6) were electroporated with 200 μM LMP1-siRNA or control GFP-siRNA. LMP1 and DAPK expression were determined by immunoblotting 48 h post-electroporation.

In vitro kinase assay

Endogenous DAPK proteins were immunoprecipitated and DAPK activity was measured as degree of phosphorylation on myosin light chain (MLC) substrate using γP^{32}-ATP. Briefly, proteins from LCLs were incubated with anti-DAPK antibodies at 4 °C overnight, followed by absorption with protein A/G beads (Calbiochem, San Diego, CA) for 3 h at 4 °C. After washing with cold NP40 lysis buffer and 1× kinase buffer (50 mM HEPES, 8 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mg/ml bovine serum albumin), beads were incubated at room temperature for 15 min in 50 μl of prepared reaction buffer containing 1×kinase buffer, 10 μM bovine CaM, 5 mM $CaCl_2$, 5 μg GST-MLC, 1 mM ATP and 10 μCi γP^{32}-ATP (GE Life Sciences, Uppsala, Sweden). Reactions were stopped by SDS sample buffer and held at 95 °C for 5 min. Supernatants were separated with SDS-PAGE and transferred to PVDF membrane. Autoradiography was performed to determine kinase activity; the same membrane was blotted with anti-DAPK antibodies. Positive controls were prepared from the lysates of DAPK-expressing HEK293T cells (pRK5F-DAPK plasmid and anti-flag antibody precipitation) (Sigma-Aldrich, St. Louis, MO).

Lentivirus knockdown system

Lentivirus shRNA clones (pLKO.1-shDAPK983 and pLKO.1-shDAPK984) and two plasmids (pMD.G and pCMV deltaR8.91) were provided by the National RNAi Core Facility at Academia Sinica, Taipei. Corresponding shRNA sequences to DAPK were 5'-CCGGCCACGTCGA-TACCTTGA AATTCTCGAGAATTTC AAGGTATCGACGTGGTTTTT-3' (pLKO.1-shDAPK 983) and 5'- CCGGCGGCACCTCTTACAATTCCATCTC-GAGATGGAATTGTAAGAG GTGCCGTTTTT-3' (pLKO.1-shDAPK 984). For lentivirus preparation, three constructs were co-transfected into HEK293T cells. Virus supernatants were collected at 24, 36, and 48 h. Following 0.22 μm filtration, viruses were aliquoted and stored until used. To generate DAPK-knockdown LCL cells, equal volumes of LCLs and lentivirus supernatant were incubated with 8 μg/ml protamine sulfate (Sigma-Aldrich) at 37 °C for 24 h. DAPK knockdown LCL cells were selected and maintained in fresh medium containing 2 μg/ml puromycin (Sigma-Aldrich). shLuc cells (pLKO.1-shLuc, a luciferase shRNA-expressing plasmid) were used as a negative control.

UV irradiation and H_2O_2 treatment

To test for stress response and DAPK activity, LCL-shLuc, LCL-shDAPK983, and LCL-shDAPK984 cells were exposed to UV or treated with oxidative H_2O_2. Briefly, 2×10^6 cells were stimulated in the presence or absence of UVC (254 nm) at 2 mJ/cm^2 using a UV Stratalinker (Stratagene, La Jolla, CA), or treated with 1 mM H_2O_2. UV-exposed cells were collected at 18 h and H_2O_2-treated cells were harvested at 24 h. Cell survival was determined using WST-1 proliferation reagent (Clontech, Mountain View, CA) following the manufacturer's instructions. Survival percentage was calculated as O.D. 450 absorbance in each batch of treated cells divided by absorbance in untreated cells.

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2011.01.032.

Acknowledgments

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